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Specific Regions of β-Globin RNA Are Resistant to Nuclease Digestion in RNA-Protein Complexes in Chicken Reticulocyte Nuclei

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The interaction between β-globin RNA and proteins in chicken reticulocyte nuclei was studied by determining the sequence of nuclease-resistant β-globin RNA. Two types of nuclease-resistant RNAs were isolated for this study: endogenous nuclease-resistant RNA from 50S heterogenous nuclear RNA-protein complexes and micrococcal nuclease-resistant nuclear RNA from whole nuclei. The nuclease-resistant regions were identified with the use of a RNA mapping method we recently developed (J. R. Patton and C.-B. Chae, J. Biol. Chem. 258:3991–3995, 1983). We found that β-globin RNA is assembled into heterogenous nuclear RNA-protein complexes in a specific manner. There are several regions of nuclease resistance in the first and third exons interrupted at regular intervals by sensitive regions. The second exon has only one nuclease-resistant region. The resistant regions range in size from 20 to 50 nucleotides. This organization may reflect a specific mode of assembly for heterogenous nuclear RNA-protein complexes.

Concomitant with the transcription of heterogenous nuclear RNA (hnRNA), the transcript is bound to proteins (14, 20) to form heterogenous nuclear RNA-protein complexes (hnRNP). When precautions are taken to limit the action of endogenous nucleases, the isolated hnRNP can be quite large (200S) and have a beaded appearance in electron micrographs (12, 21, 31). When either endogenous or exogenous nucleases are allowed to act on the hnRNP, particles between 30 and 50S are released. These core particles are considered to be the basic structural units of hnRNP (13, 16), and although there may be as many as 40 protein components of these particles, there are usually only six major proteins, with molecular weights between 30,000 and 45,000 (3, 10, 16, 26, 33). These major proteins can be cross-linked by UV light to RNA in situ (5); therefore, the interaction between hnRNA and protein is apparently close.

Since the assembly of hnRNA into hnRNP takes place while transcription is occurring, the subsequent steps in the maturation of the RNA must take place with the proteins attached to the transcript. These proteins might serve to protect the RNA from digestion by nucleases. Alternatively, these proteins could stabilize (1) or actually induce (35) an RNA structure which is favorable for processing or transport. A central question regarding the structure of hnRNP is whether the proteins are bound to the RNA in a specific manner. Beyer et al. (4) provided visual evidence, in the form of electron micrographs, that hnRNP are assembled nonrandomly. There is biochemical evidence supporting the specific assembly of hnRNP (23, 28, 32, 34). In all of the related biochemical studies, nuclease digestion combined with analysis of nuclease-resistant RNA has been the basic approach, with the assumption that interaction with proteins affords resistance to nuclease digestion. Solution hybridization (28) and Southern blot hybridization (22, 23, 32) have been used to characterize the nuclease-resistant RNA. However, sensitivity of these methods is low and at best can only define a general area that is resistant to nuclease.

We found that β-globin RNA is assembled into hnRNP in a specific manner in chicken reticulocytes. Nuclease-resistant regions have been mapped, at the resolution of nucleotides, by the new RNA mapping method we have recently developed (25). There are extensive regions of nuclease resistance in the first and third exons, interrupted at regular intervals by sensitive regions. The second exon has only one nuclease-resistant region. We discuss how this organization could reflect a specific mode of assembly for hnRNP.

MATERIALS AND METHODS

Isolation of nuclei and polyribosomes. White Leghorn chickens (1 to 2 kg) were injected intramuscularly for 7 days with phenylhydrazine (10 mg/kg per day). Blood was collected in 0.14 M NaCl–5 mM KCl–1.5 mM $MgCl_2$ with 0.1 mg of heparin per ml. The reticulocytes were used for the isolation of polysomes and nuclei as described previously (7).

Preparation of RNA from 50S hnRNP. The 50S hnRNP released by endogenous nuclease were isolated from reticulocyte nuclei as described by Maundrell and Scherrer (17) and Huang and Chae (9). 50S hnRNP was purified on a 15 to 30% sucrose gradient, and the RNA from the hnRNP was purified as described previously (9). The gradient profile and the protein patterns of the 50S hnRNP preparation were also reported previously (9). The particle had previously been assigned a sedimentation value of 40S (9) but is actually 50S.

Digestion of nuclei and isolation of MNR-RNA. Micrococcal nuclease-resistant RNA (MNR-RNA) was isolated as follows. Nuclei from chicken reticulocytes were washed once with 10 mM Tris (pH 7.5)–30 mM KCl–1 mM EDTA–0.25 M sucrose and twice with TKM (10 mM Tris [pH 7.5], 30 mM KCl, 2 mM $MgCl_2$) plus 0.25 M sucrose. The nuclei were suspended in TKM plus 0.25 M sucrose at 1 mg/ml (in nucleic acids) and digested with micrococcal nuclease (500 U/ml) for 1 h at 22°C with occasional gentle shaking. Phenylmethylsulfonyl fluoride (0.1 mM) was present as a protease inhibitor. EDTA was added to 5 mM to stop the reaction, and the nuclei were pelleted. The pellet was

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suspended in 0.1% sodium dodecyl sulfate–10 mM Tris (pH 7.5)–50 mM NaCl–1 mM EDTA–10 mM vanadyl-adenosine complex (a ribonuclease inhibitor [2]) and digested at 37°C with 200 μg of proteinase K per ml for 1 h. The solution was extracted three times with phenol and once with chloroform and precipitated with ethanol at −20°C. After ethanol precipitation, the pellet was suspended in 10 mM Tris (pH 7.5)–5 mM $MgCl_2$ and digested with DNase I (RNase free; 60 U/ml; Worthington Diagnostics) for 1 h at 37°C. The sample was extracted twice with phenol and once with chloroform and precipitated with ethanol.

Preparation of total nuclear RNA. Nuclei were washed as described above, and the concentration of nucleic acid was adjusted to 1 mg/ml in TKM plus 0.25 M sucrose. The nuclei were digested with DNase I (RNase free, 10 U/ml) for 1 h at 22°C, and the reaction was stopped with EDTA. RNA was extracted as described for total nuclease-resistant RNA.

Sizing of β-globin RNA fragments. The replicating form (RF) of M13mp7 recombinant phage containing β-globin gene fragments was bound to nitrocellulose filters (20 μg per 25-mm filter). Samples of nuclease-resistant RNAs were labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP and hybridized to the filter-bound DNA. The β-globin RNA sequences were eluted from the filters as described by Huang and Chae (9). The eluted RNAs were electrophoresed on 12% polyacrylamide gels in 8 M urea (18) and autoradiographed.

Preparation of micrococcal nuclease-digested protein-free polysomal RNA. Polysomal RNA (1 mg/ml) was digested for various times at 22°C with micrococcal nuclease (150 U/ml) in TKM plus 0.25 M sucrose and 1 mM $CaCl_2$. A sample was removed just before the addition of nuclease and incubated for 64 min as a control. After digestion, the samples were made 5 mM EDTA, extracted three times with phenol-chloroform (1:1) and once with chloroform, and precipitated with ethanol. The size of the β-globin RNA fragments in these digested samples was determined as described above.

Preparation of single-stranded DNA probes. DNA fragments containing each of the three coding blocks of the adult chicken β-globin gene were inserted in the *Hin*cII or *Sal*I site of M13mp7RF. Clones containing the first (mβG6) and third (mβG7) coding regions have already been described (25). The second coding region from the *Acc*I to the *Ava*I site (see Fig. 3) was also cloned into the *Hin*cII site of M13mp7RF. The orientation of the fragments was such that the phage DNA would hybridize to globin RNA.

The single-stranded β-globin gene fragment complimentary to β-globin RNA was excised from the recombinant phage DNA with *Bam*HI (24) and labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (7,000 Ci/mmol; ICN) (15). The approximate radiospecific activity was calculated from the amount of label incorporated into the DNA fragments. The amount of DNA was estimated from the intensity of staining with ethidium bromide. [^{3}H]cDNA was prepared from polyadenylic acid [poly(A)]-containing RNA isolated from chicken reticulocyte polysomes by the method of Friedman and Rosbash (6).

RNA mapping. Total nuclease-resistant RNA and RNA from 50S hnRNP were mapped on the three β-globin gene probes by the method of Patton and Chae (25). One of the single-stranded DNA probes (20,000 cpm; ~5 × 10^6 to 10 × 10^6 cpm/μg) was mixed with 50 μg of RNA (actual amounts of chicken nuclear RNA are given in figures) in 0.4 M NaCl–10 mM Tris (pH 7.5)–1 mM EDTA in a final volume of 50 μl. Since 50 μg of chicken nuclear RNA was not used for every experiment, yeast RNA was used to adjust the RNA concentration. The tubes were heated at 90°C for 2 min and incubated for 16 h at 68°C. The samples were precipitated with 2.5 volumes of ethanol. The dried pellet was taken up in 0.4 ml of 30 mM sodium acetate (pH 4.6)–50 mM NaCl–1 mM $ZnSO_4$–5% glycerol and digested with nuclease S1 (6 U; Bethesda Research Laboratories, Inc.) or 6 U of mung bean nuclease (P-L Biochemicals) at 37°C for 1 min. The reactions were stopped by adding EDTA to 10 mM, and the nucleic acids were precipitated with ethanol after adjusting the sodium acetate concentration of 0.3 M. The pellet was treated with 0.3 N NaOH at 68°C for 30 min. The reaction was neutralized with 3 N hydrochloric acid plus 0.1 M Tris (pH 7.5). The probe was precipitated with 2.5 volumes of ethanol in the presence of 5 μg of carrier yeast RNA. The dried pellet was taken up in formamide loading buffer (18), heated at 90°C, and loaded onto a sequencing gel.

RNA excess hybridization in solution. Hybridization samples were set up as described above for RNA mapping with the exception that [^{3}H]cDNA was used as the probe. After hybridization, the reaction was taken up in 1 ml of S1 digestion buffer (described above), and a portion (800 μl) was digested with 20 U of S1 nuclease for 1 h at 37°C. The rest of the sample was used as an undigested control. The S1-resistant nucleic acids were precipitated with trichloroacetic acid, and radioactivity was counted in a Beckman scintillation counter.

DNA sequencing. The procedure of Maxam and Gilbert (18) was used to sequence the 5′-end-labeled DNA fragments and to prepare the acrylamide gels (0.3, 330, 400 mm). *Hin*fI-cut M13mp7RF was end labeled as described and used as a marker.

RESULTS

The interaction between β-globin RNA and proteins in chicken reticulocyte nuclei was studied by determining the sequence of nuclease-resistant β-globin RNA. The RNA that is resistant to digestion is assumed to be protected by protein (see below). Two types of nuclease-resistant RNA were isolated for this study: endogenous nuclease-resistant RNA from 50S hnRNP and MNR-RNA from whole nuclei.

The 50S particle is considered the basic unit of hnRNP (13, 16), and since it is the result of endogenous nuclease digestion, it was a convenient source of nuclease-resistant RNA. The endogenous nucleases that digest the RNA in hnRNP are not characterized in terms of specificity for single- or double-stranded RNA. Since it is impossible to differentiate between resistant RNA that was protected from digestion by proteins and RNA that was resistant to digestion due to RNA secondary structure, we also isolated MNR-RNA for comparison. Micrococcal nuclease digests both single- and double-stranded RNA and, at the high concentration used in this study, leaves only the RNA resistant to nuclease attack due to bound proteins.

We have shown earlier that the 50S particles, isolated by sucrose gradient centrifugation of the hnRNP extracted from chicken reticulocyte nuclei, have the characteristic set of proteins in the molecular weight range of 30,000 to 45,000 (9). The sizes of resistant β-globin RNA fragments are small, between 20 and 85 nucleotides, and the sizes of fragments from the different exons of β-globin RNA do not vary considerably (data not shown; see above for details). With MNR-RNA, the sizes of β-globin RNA fragments are again small, ranging from 19 to 60 nucleotides. Although there are some differences in the patterns of resistant RNA fragments from each exon of β-globin RNA, the differences are not significant (data not shown). The patterns are slightly dif-

ferent for β-globin RNA fragments generated by the endogenous enzyme (RNA from 50S hnRNP) and micrococcal nuclease (MNR-RNA), probably due to a difference in the specificities or the modes of action of the two enzymes.

These sizing experiments told us little about whether specific β-globin RNA sequences were resistant to digestion. As a preliminary experiment to test the possible protection of specific regions of β-globin RNA, increasing amounts of MNR-RNA were hybridized to [^{3}H]cDNA prepared from reticulocyte polysomal poly(A)-containing RNA (Fig. 1). If specific sequences are resistant or sensitive to nuclease, then the maximum percent hybridization will be less than 100%, depending on what portion of the RNA is resistant. At a low RNA concentration (an RNA/probe ratio of about 10), the hybridization curve levels off at 50%, suggesting that specific sequences are resistant to digestion (Fig. 1). After initially leveling off at 50%, the curve continues to rise gradually with increasing RNA concentration. This suggested to us that the major component of MNR-RNA might be derived from specific regions but that the MNR-RNA also contains minor components of RNA which are most likely derived from different types of RNA-protein complexes. When, as a control, protein-free nuclear RNA was digested with micrococcal nuclease under the same conditions as those used for whole nuclei, the RNA did not hybridize to the cDNA probe (not shown here), indicating that bound proteins are responsible for the nuclease resistance of the RNA.

Although it would be impossible to assess the hybridization data in relation to β-globin RNA in particular, the results suggested that there could be specific nuclease-resistant regions in nuclear RNA, including β-globin RNA. So we were encouraged to obtain information on the sequences of the resistant β-globin RNA fragments from 50S hnRNP RNA and MNR-RNA.

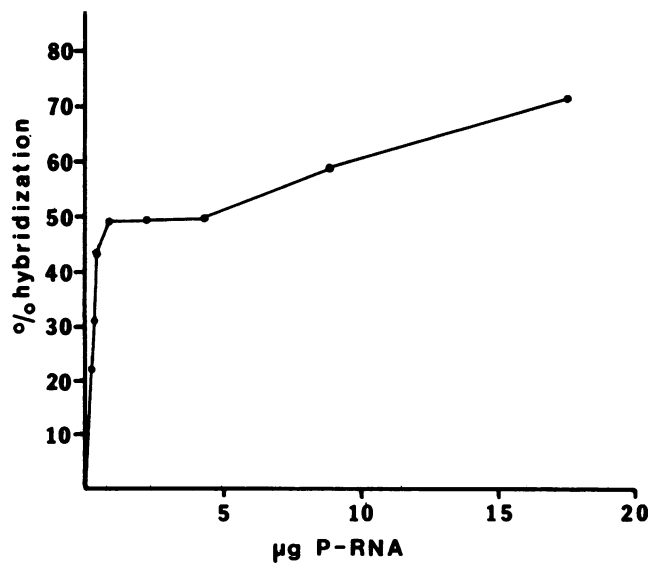

FIG. 1. Solution hybridization of total nuclease-protected nuclear RNA with a globin cDNA probe. [^{3}H]cDNA made from poly(A)-containing RNA from chicken reticulocyte polysomes was hybridized to MNR-RNA, and the percent hybridization was determined as described in the text. The percent hybridization was calculated with the level of hybridization of total polysomal RNA defined as 100%.

β-Globin RNA is one of the abundant mRNAs in the cytoplasm of reticulocytes, but in the nucleus it constitutes only approximately 0.1% of the total RNA (30). In 50S hnRNP RNA, the value is higher, but the content of β-globin RNA is still less than 1% of the total RNA (17; unpublished data). We needed a method of studying the resistant β-globin RNA sequences without having to purify these minor components of the total RNA. A RNA mapping method we recently developed (25) solves this problem and was used to determine nuclease-sensitive and -resistant regions on β-globin RNA.

In this method, RNA samples are hybridized to end-labeled single-stranded chicken β-globin DNA probes. The RNA-DNA hybrid is mildly digested with a single-strand-specific nuclease, such as nuclease S1, and the products are electrophoresed on a high-resolution polyacrylamide gel. An area of the probe hybridized to RNA will be resistant to S1 digestion and will appear as blank on an autoradiograph compared with a control that has no β-globin RNA in the hybridization reaction. To identify these regions, the probe cleaved with the base-specific reagents of Maxam and Gilbert (18) is electrophoresed concomitantly with the samples. The major component of nuclease-resistant RNA can be mapped at low RNA/probe ratios (Fig. 1). Since the specific activity of the probe cannot be accurately determined, we varied the amount of nuclease-resistant RNA used in the hybridization reactions. For this report, we mapped only the nuclease-resistant regions found in the exons. Since there is 10 times more mature β-globin RNA than precursor β-globin in the nucleus (30), at a low RNA/probe ratio we only examined the nuclease-resistant RNAs derived from mature globin RNA.

Single-stranded fragments containing the β-globin exons were isolated from the recombinant phage DNA of M13mp7. The clones containing the first and third exons have been described previously (25). Most of the second exon, from the *Acc*I to the *Ava*I site (see Fig. 3), was cloned into the *Hin*cII site of M13mp7RF. The resulting fragment is missing the first 13 and last 14 nucleotides of the second exon. The single-stranded globin inserts were isolated from phage DNA by digestion with *Bam*HI as described previously (24). The fragments were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (15) and used in the mapping experiments.

Nuclease-resistant regions in the first exon. Figure 2 shows the results of a mapping experiment with a globin gene probe from the first exon. The probe contains approximately 200 bases of the 5′-flanking sequence, the first exon, and 26 bases of the first intron. When polysomal RNA or total nuclear RNA (Fig. 2, lanes a and b) was included in the hybridization reaction, the entire exon from the transcription initiation site (CAP) to the bottom of the lane (splice donor site) appeared blank compared with the control, yeast RNA (lane c). Both of these RNAs contain intact β-globin RNA sequences and should hybridize to the entire exon sequence of the probe. However, in the nontranscribed flanking region, the probe was digested by S1 to the same extent as was the yeast RNA sample (Fig. 2, lane c). When increasing amounts of MNR-RNA were included in the hybridization reaction (Fig. 2, lanes d through g), the intensities of several regions (marked with brackets) appeared reduced compared with the control lane (lane c). These regions, where RNA was hybridized to the probe, are apparent at low MNR-RNA/probe ratios (Fig. 2, lanes d and e). Some regions were still sensitive to S1 digestion, indicating that these regions were not hybridized to RNA. As the concentration of resistant RNA increased (Fig. 2, lanes f and

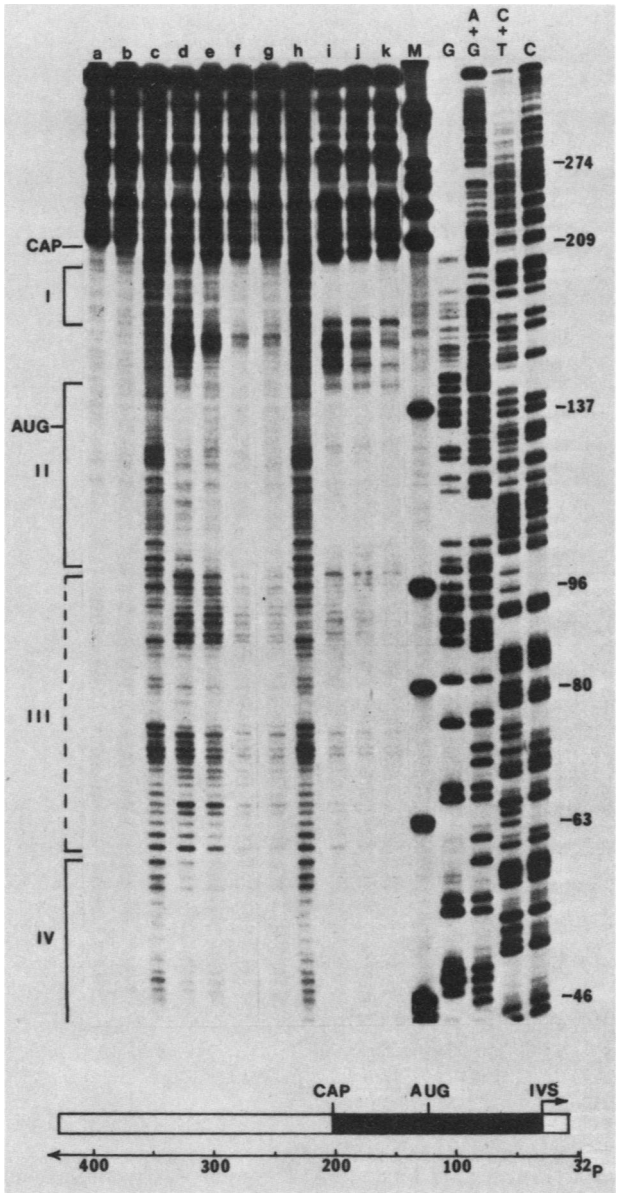

FIG. 2. Mapping of nuclease-sensitive and -resistant regions of β-globin RNA on the first coding block of the β-globin gene. (Top) The 430-base probe from mβG6 containing the first coding region of the chicken β-globin gene (see scale map at bottom of figure) was hybridized with total polysomal RNA (lane a); undegraded nuclear RNA (lane b); yeast tRNA (lanes c and h); 1, 3, 10, and 25 μg of MNR-RNA (lanes d through g, respectively); 0.15, 0.3, and 10 μg of RNA from 50S hnRNP, (lanes i through k, respectively). Sizes of molecular weight markers (M) are shown on the right in bases. Lanes G, A+G, C+T, and C, Base-specific cleavage sequencing reactions. CAP, Transcription initiation site; AUG, translation start codon; brackets define regions discussed in the text. (Bottom) Scale map of the probe, indicating the position of the label. IVS, Intervening sequence border.

g), most of the exon was hybridized to RNA. This may be due to the hybridization of minor components of the resistant RNA to the regions of the probe initially unoccupied by the abundant resistant RNA. These minor components are most likely derived from different RNP (Fig. 1; see below). The regions of the probe that were hybridized and not hybridized to nuclease-resistant RNA were mapped with the aid of DNA sequencing and size markers (see Fig. 6 for a summary of results obtained with low RNA concentrations).

When RNA from 50S hnRNP was used in the hybridization reaction (Fig. 2, lanes i through k), most of the hybridized and nonhybridized regions of the probe were similar to those seen with MNR-RNA. The exception is region III of the probe, where RNA from 50S hnRNP (lane i and j) hybridizes but not completely. This region was not hybridized when MNR-RNA was used in the hybridization reaction.

Nuclease-resistant regions in the second exon. The same type of mapping experiment was done with the second exon probe. Since this probe does not contain any intervening sequence, the entire probe was hybridized to polysomal RNA (Fig. 3B, lane a).

When increasing amounts of RNA from 50S hnRNP were used in the hybridization reactions (Fig. 3A, lanes b through f), only one region of the probe (region I), was hybridized to 50S hnRNP at low and moderate RNA concentrations (lanes b through d). Region II, between 13 and 41 nucleotides from the labeled end, was hybridized to RNA only at high RNA concentration (Fig. 3A, lane f). The extreme 5′ end of the probe was not resolved well in our gel system, and it is not clear whether 50S hnRNP RNA hybridizes to this region.

The single-stranded probe was cut unevenly by S1 even when no β-globin RNA was included in the hybridization (Fig. 3A, lane a; Fig. 3B, lane b). Some areas, for example, the question-marked bracket between 96 and 137 bases from the labeled end, were not cut at all by S1. These blank regions could be due either to S1 sequence specificity or to the secondary structure of the probe. In these areas, it is not possible to tell whether resistant RNA was hybridized to the probe.

MNR-RNA and 50S hnRNP RNA hybridized to essentially the same region of the probe (Fig. 3B, lanes c through f). Again, region II, between 13 and 42 nucleotides from the labeled end of the probe, was hybridized only at high RNA concentrations (Fig. 3B, lanes d and e). Also shown in Fig. 3B is the addition of MNR-RNA immediately before S1 digestion (lane f). This control demonstrates that the resistant RNA does not simply inhibit S1 digestion of the probe but that the blank regions are the result of hybridization of the probe with RNA. (See Fig. 6 for a summary of the regions of this probe that hybridized to nuclease-resistant RNA; the regions which cannot be evaluated with confidence are indicated by question marks.)

Nuclease-resistant regions in the third exon. Figure 4 shows the results of a RNA mapping experiment with a globin gene probe from the third exon. This probe contains 331 bases of the second intervening sequence, the third exon, and 62 bases of sequence 3′ to the poly(A) addition site. When polysomal RNA or total nuclear RNA was included in the hybridization reaction (Fig. 4B, lanes a and b), the entire exon from the intervening sequence border (marked IVS) to the poly(A) addition site was hybridized (compare with control yeast RNA in lane c).

When RNA from 50S hnRNP was used in the hybridization reaction (Fig. 4A, lanes b and c), several regions of the probe were hybridized, and they are marked by brackets I through IV in the figure. The regions near the splice site (marked IVS) were mapped from an autoradiograph of a gel that was electrophoresed for a longer time than the gel in Fig. 4A (gel not shown). This allowed precise assignment of sequence to the regions (see Fig. 6 for a summary of the locations and lengths of these nuclease-resistant regions).

When MNR-RNA was used in hybridization reactions

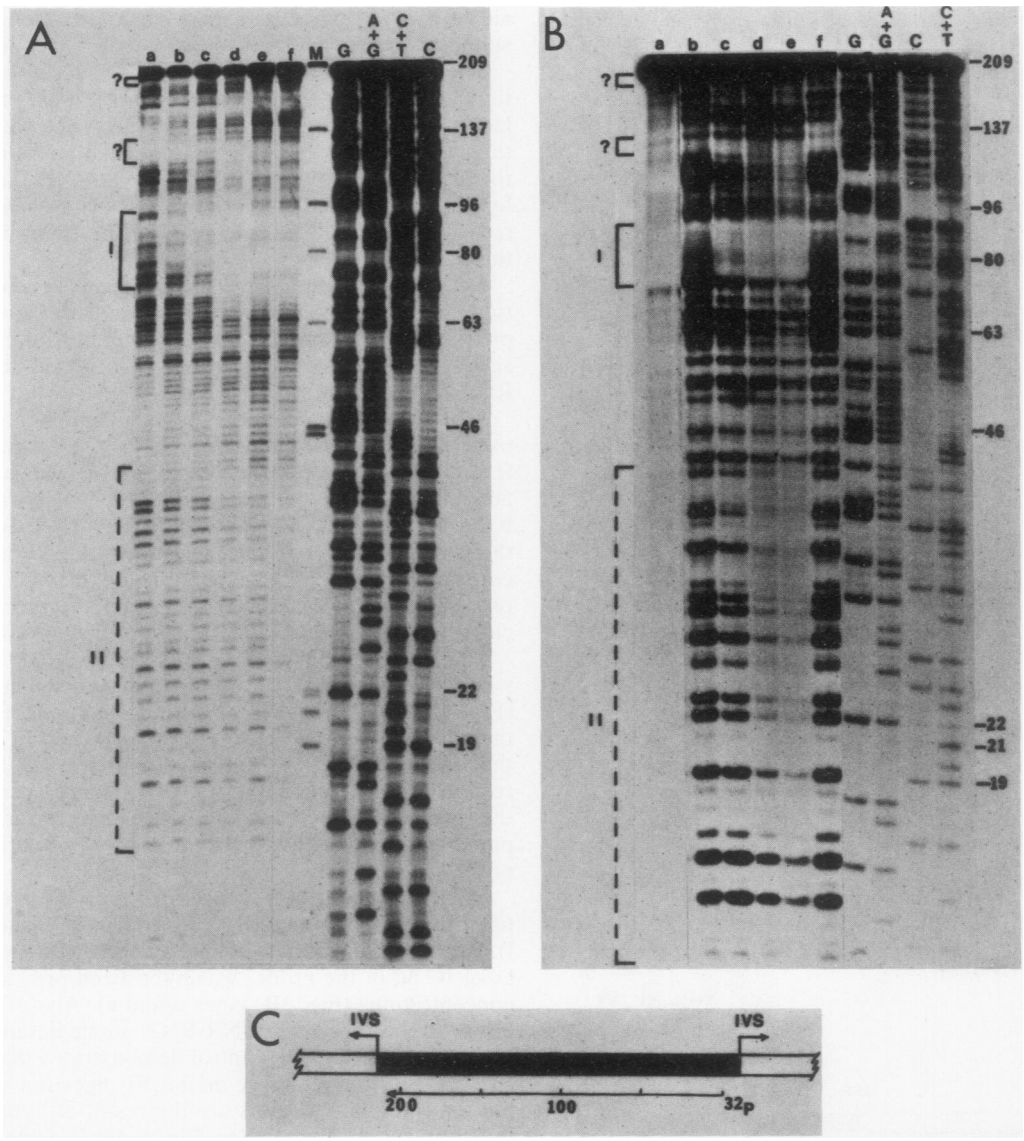

FIG. 3. Mapping of nuclease-sensitive and -resistant regions in RNA on the second coding block of β-globin gene. The probe from mβG12 containing most of the second coding block of the chicken β-globin gene (see scale map [C]) was used. (A) The probe was hybridized with the following: yeast RNA (lane a); 0.15, 0.30, 0.60, 1.2, and 5.0 μg of RNA from 50S hnRNP (lanes b through f). (B) The probe was hybridized with the following: total polysomal RNA (lane a); 1, 3, 8, and 10 μg of MNR-RNA (lanes b through e, respectively); 10 μg of MNR-RNA heated at 90°C before mung bean nuclease digestion (lane f). Lanes G, A+G, C, and C+T, DNA sequencing reactions. Positions of molecular weight markers (lane M) are indicated on the right in bases; brackets define regions discussed in the text. (C) Scale map of the probe, indicating the position of the label.

(Fig. 4B, lanes d through g), slightly different results were obtained. Region I was the same as was seen with 50S hnRNP. Region II, seen with 50S hnRNP RNA, was shifted slightly towards the labeled end of the probe when MNR-RNA was used. This region was not completely hybridized even at high concentrations of MNR-RNA. Region III, seen with RNA from 50S hnRNP, was not apparent when the concentration of MNR-RNA was low (Fig. 4B, lane d). At higher RNA concentrations, an area that corresponds to region III in Fig. 4A was hybridized to MNR-RNA, and the nonhybridized region became the same size as that seen with RNA from 50S hnRNP (lane f). Region IV, initially small at low MNR-RNA concentrations, widened at higher RNA concentrations (lane f) (see Fig. 6 for a summary of the similarities and differences between MNR-RNA and 50S hnRNP RNA).

Control experiments. The results shown in the previous figures suggest that the RNA from 50S hnRNP and MNR-RNA are derived from specific regions of β-globin RNA. Thus, specific regions of β-globin RNA are resistant to endogenous nuclease in the case of 50S hnRNP and to micrococcal nuclease in whole nuclei. This is most likely due to protection of the RNA from nuclease digestion by bound proteins. Results from several control experiments support this conclusion. For example, when total protein-free nuclear RNA was digested by micrococcal nuclease under the identical conditions as the digestion of whole nuclei, the resulting RNA fragments did not hybridize to the gene probes.

To test the possible sequence specificity of micrococcal nuclease, protein-free polysomal RNA was digested by micrococcal nuclease until the β-globin RNA was the same

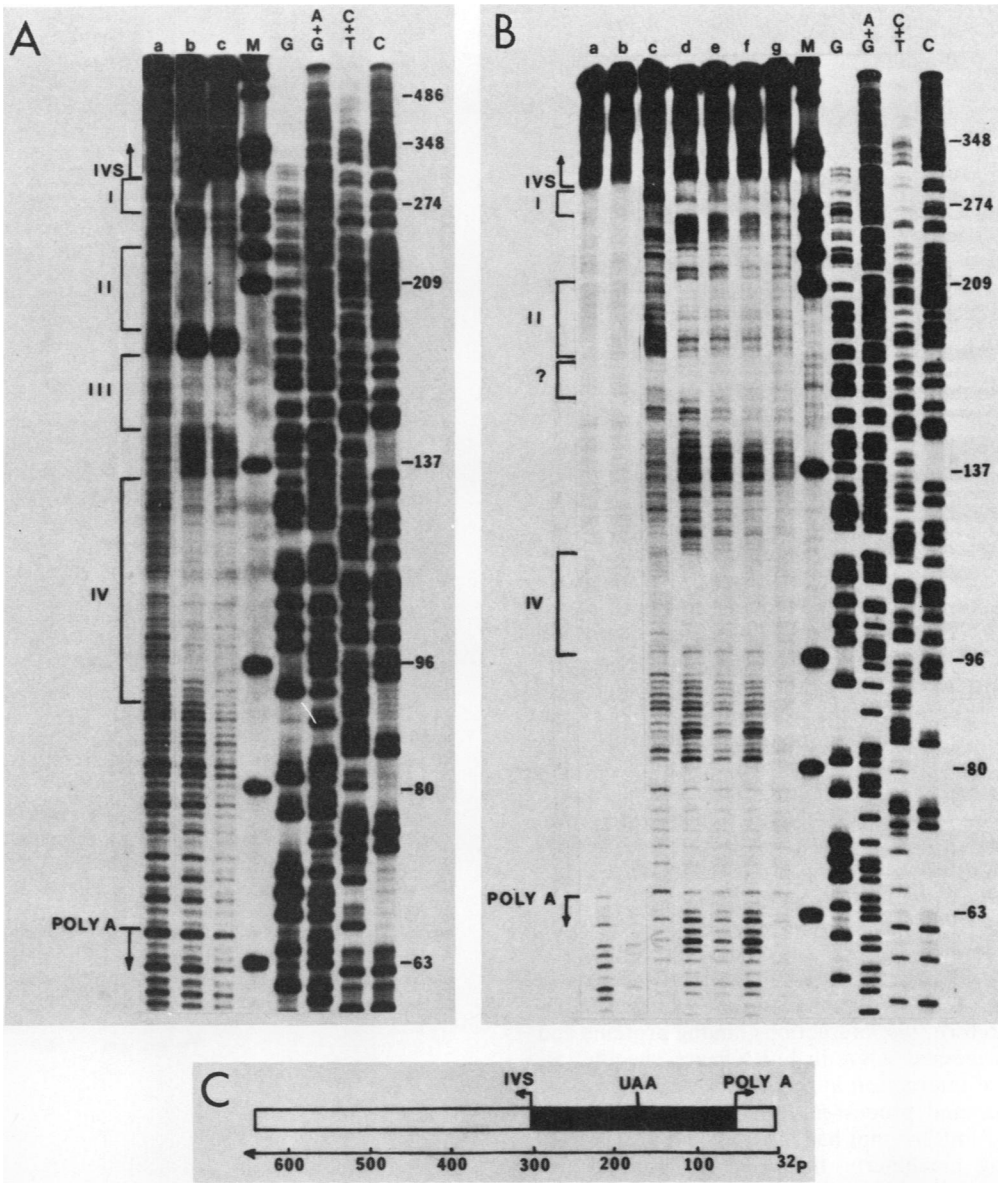

FIG. 4. Mapping of nuclease-sensitive and -resistant regions of β-globin RNA on the third coding block of the β-globin gene. The 634-base probe (mβG7) containing the third coding region (see scale map at bottom) was used. (A) The probe was hybridized with the following: yeast tRNA (lane a); 1 and 10 μg of RNA from 50S hnRNP (lanes b and c). (B) The probe was hybridized with the following: polysomal RNA (lane a); undergraded nuclear RNA (lane b); yeast tRNA (lane c); 3, 10, 15, and 25 μg of MNR-RNA (lanes d through g, respectively). Sizes of molecular weight markers (lane M) are shown on the right in bases. Lanes G, A+G, C+T, and C, Base-specific sequencing reactions; IVS, the splice acceptor site; POLY A, the poly(A) addition site; brackets define regions discussed in the text. (C) Scale map of the probe, indicating the position of the label.

size as β-globin RNA from MNR-RNA (19 to 60 nucleotides, see above for determination of the size of β-globin RNA). The resistant RNA was used in a RNA mapping experiment with the third exon probe (Fig. 5). The RNA in lane d of Fig. 5 produced a β-globin RNA of about the same size as the β-globin RNA in MNR-RNA. The RNA did not hybridize to any specific regions of the probe (Fig. 5, lane d) and produced essentially the same results as did the nonglobin control (lane f). However, the overall intensity of bands was reduced due to the hybridization of globin RNA fragments generated by random digestion by micrococcal nuclease. The exception is the absence of bands in the region indicated by a bracket in the polysomal RNA sample (Fig. 5, lane d). Therefore, micrococcal nuclease generates a set of random fragments from β-globin RNA. It appears that the results obtained with the nuclease-resistant RNA from whole nuclei and also the RNA from 50S hnRNP are due to the protection of specific regions of RNA by proteins and do not reflect any specificity of the nuclease for certain sequences.

Protein translocation did not occur during nuclease digestiosn. Whole nuclei were exposed to UV under conditions that efficiently cross-link hnRNP (19). By using cesium sulfate gradients, it was found that practically all of the nuclear RNA was cross-linked to proteins. The nuclease-re-

sistant RNA obtained from UV-cross-linked nuclei produced essentially the same pattern of hybridization as MNR-RNA (not shown here).

DISCUSSION

In this report, we investigated the nuclease-sensitive and -resistant regions of β-globin RNA in whole nuclei and 50S hnRNP of chicken reticulocytes to obtain information on the possible specific assembly of hnRNP. Examination of the major component of nuclease-resistant β-globin RNA fragments shows that there are definitely specific nuclease-resistant sites on β-globin RNA. This is probably due to the specific binding of proteins to β-globin RNA in light of the control experiments described above. There are several resistant regions in the first and third exons of β-globin RNA but only one resistant region in the second exon. MNR-RNA and RNA from 50S hnRNP give very similar overall patterns (Fig. 6). Our results indicate that the bulk of β-globin RNA is present in the form of hnRNP in the nucleus. The minor differences in the pattern of nuclease-resistant regions between the two RNA preparations could be due to the possible difference in the action of micrococcal nuclease used for whole nuclei and the endogenous nuclease which produced 50S hnRNP. Another possibility is limited protein rearrangement during the preparation of 50S hnRNP.

Our results most likely reflect the specific assembly of hnRNP containing the processed β-globin RNA. We previously found that the amount of mature β-globin RNA is greater than 90% of total β-globin RNA (30), and our preliminary experiments with 50S hnRNP show that it takes about 10 times more 50S hnRNP RNA to hybridize to intron probes than it takes to hybridize to exon probes. Thus, in nuclei there are hnRNP containing processed RNA as well as hnRNP containing precursor β-globin RNA (27). Preliminary experiments show that there is one nuclease-resistant region in the middle of the first intron and there are two nuclease-resistant areas in the first 400 bases of the second intron in 50S hnRNP (J. R. Patton and C.-B. Chae, unpublished data). Therefore, the interactions among proteins and precursor and processed RNA in hnRNP are specific, although the sites of interaction in the exons may not be the same in precursor and processed RNA. Inasmuch as the precursor RNA in hnRNP, not naked RNA, is the substrate for RNA splicing machinery, the specific assembly we observe with β-globin RNA suggests that the proteins in hnRNP most likely play an important role in RNA splicing. A comparison of the protein-binding sites on precursor and processed RNA may provide important information with regard to the role of hnRNP in RNA splicing.

The significance of specific assembly of hnRNP is not clear at the present time. Also, the mechanism of specific assembly of hnRNP is unknown. Considering the similarity of hnRNP proteins in various tissues and among species (3, 10, 16), it is difficult to envision that the hnRNP proteins recognize specific RNA sequences for binding. The number of proteins, each having a separate sequence specificity, would have to be quite large, given the sequence complexity of RNA transcribed in the reticulocyte nucleus. A more attractive model for the specific assembly of hnRNP is the recognition of the 5′ end of precursor RNA by a hnRNP protein and subsequent self-assembly of the rest of the particle in a specific order irrespective of the RNA sequence or the secondary structure of the RNA. Such a mode of assembly would result in protection of specific regions of the transcript from nuclease digestion, since all transcripts from the same gene would be assembled in the same way. After

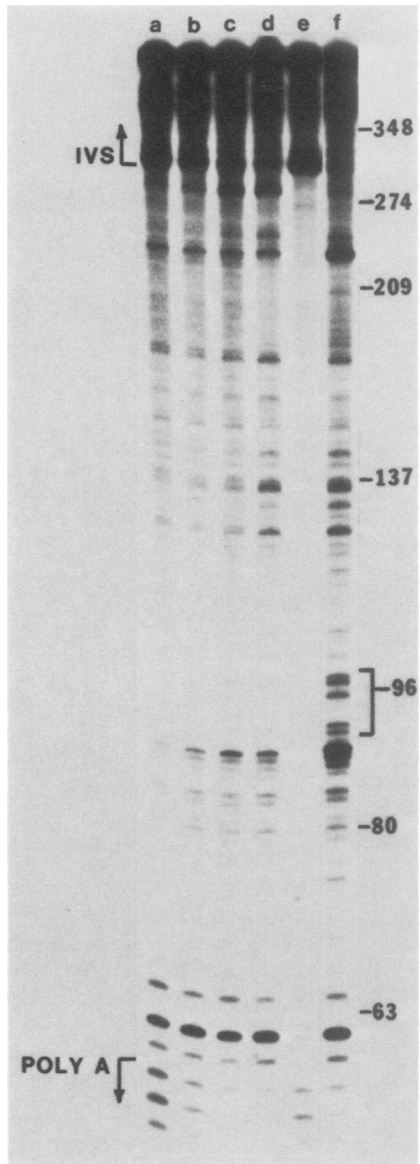

FIG. 5. Test for micrococcal nuclease sequence specificity on protein-free globin RNA. The probe from the third coding block was hybridized with 8 μg of total polysomal RNA digested with the following: micrococcal nuclease (see the text) for 8, 16, 32, and 64 min (lanes a through d, respectively); 8 μg of polysomal RNA incubated without micrococcal nuclease (lane e); yeast tRNA (lane f). Brackets define regions discussed in the text, and molecular weight sizes are shown on the right in bases. The 64-min sample (lane d) produced about the same size β-globin RNA (20 to 60 nucleotides) as the β-globin RNA in MNR-RNA (see the text for determination of the size).

RNA splicing, the proteins may realign along the RNA to maintain the overall hnRNP structure. The nuclease-resistant areas from α-globin RNA in 50S hnRNP RNA are now being mapped to test this model. We are also forming β-globin RNP in vitro, by using nuclear extracts and in vitro-transcribed precursor RNA, to study the RNP structure in the absence of spliced RNA.

Concerning the model, it is of interest to note the recent reports of the requirement of a cap structure for efficient splicing in vitro, along with a lag time from the addition of

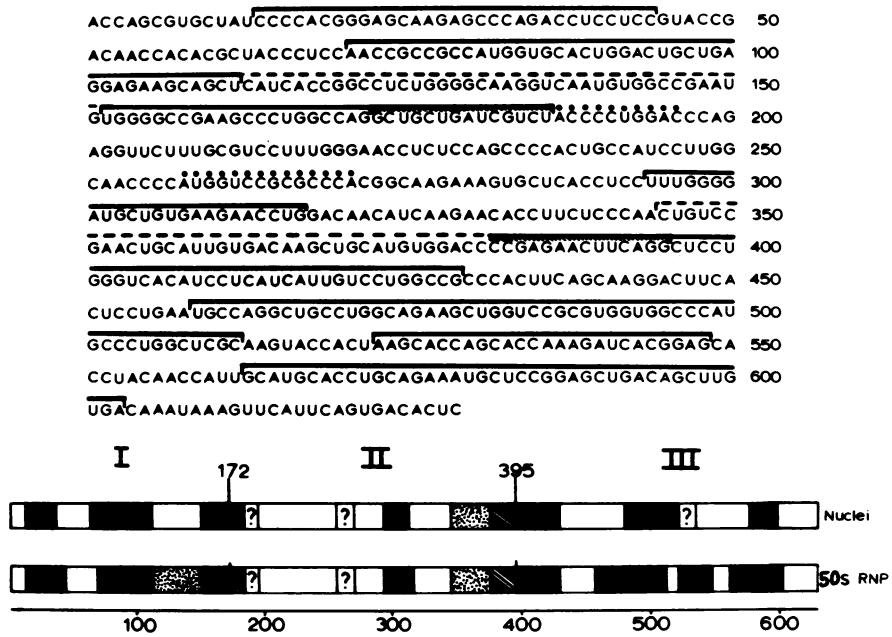

FIG. 6. Summary of mapping results. The β-globin mRNA sequence (29) with the regions of nuclease resistance in 50S hnRNP are given at the top of the figure. Solid lines denote regions of nuclease resistance; dashed lines define regions of slight sensitivity; slanted lines denote nucleotides not in the second coding block probe; and dots indicate the regions which were difficult to evaluate (indicated by question marks in the diagram below). The results for whole nuclei and 50S hnRNP are presented diagrammatically below the sequence. Solid regions denote nuclease resistance; stipled regions denote slight sensitivity; slanted lines denote nucleotides not in the second coding block probe; blanks denote nuclease-sensitive regions; and question marks denote regions in which the sensitivity to nuclease was difficult to determine. The three coding blocks of the β-globin gene are indicated by roman numerals. The positions corresponding to the last base of the first coding block (172 nucleotides) and the last nucleotide of the second coding block (395 nucleotides) are indicated.

exogenous RNA to the reaction and the detection of splicing in vitro (8, 11). The lag was thought to be the time necessary for assembly of hnRNP in cell-free nuclear extracts (8, 11). The cap structure is required for RNA splicing (8, 11) and may also be essential for the correct assembly of the hnRNP.

Several groups have looked for evidence of specific assembly of hnRNP by Southern blot hybridization of labeled nuclease-resistant RNA from hnRNP to restriction fragments of genes. Ohlsson et al. (23) reported RNase A-resistant RNA in the regions flanking splice junctions in adenovirus gene transcripts. Stevenin et al. (34) obtained evidence for nonrandom assembly of hnRNP containing adenovirus gene transcripts and possible rearrangements of proteins during processing. Munroe (22), however, was unable to detect clearly resistant or sensitive regions in adenovirus gene transcripts. As we have shown in this report, the space between resistant and sensitive regions is in the range of 20 to 50 bases, and Southern blot hybridization of restriction digests of genes does not provide sufficient resolution to map the narrow boundaries of these regions. Also, a hybridization condition which distinguishes between the nuclease-resistant RNA populations derived from precursor and processed RNA may be necessary.

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